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(71) Applicant

Yeda Research and Development Company Limited,  
(Israel),  
PO Box 95, Rehovot, Israel

(72) Inventors

Michael Sela,  
Ruth Arnon,  
Chaim O. Jacob

(74) Agent and/or Address for Service

Alan Tromans & Co.,  
7 Seymour Road, Finchley, London N3 2NG

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(56) Documents cited

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EP A1 0093652

(58) Field of search

C3H

(54) Vaccines

(57) A synthetic vaccine against cholera and against heat-labile toxin of E. Coli comprising a conjugate of a high-molecular weight carrier with a synthetic polypeptide corresponding to part of the sequence of Subunit B of natural cholera toxin. The sequences of choice are those corresponding to sequences 45 to 64, 50 to 64 and 8 to 20 of Subunit B, or slightly modified sequences, similar to the above.

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Primary Structure of Subunit B

Thr - Pro - Gln - Asn - Ile - Thr - Asp - Leu - Cys - Ala - Glu - Tyr - His - Asn -  
 \_\_\_\_\_  
 20  
 Thr - Gln - Ile - His - Thr - Leu - Asn - Asn - Lys - Ile - Phe - Ser - Tyr - Thr -  
 \_\_\_\_\_  
 30  
 Glu - Ser - Leu - Ala - Gly - Lys - Arg - Glu - Met - Ala - Ile - Thr - Phe -  
 \_\_\_\_\_  
 40  
 Lys - Asn - Gly - Ala - Thr - Phe - Glu - Val - Glu - Val - Pro - Gly - Ser - Gin -  
 \_\_\_\_\_  
 50  
 His - Ile - Asp - Ser - Gln - Lys - Ala - Ile - Glu - Arg - Met - Lys - Asn -  
 \_\_\_\_\_  
 60  
 His - Ile - Asp - Ser - Gln - Lys - Ala - Ile - Glu - Arg - Met - Lys - Asn -  
 \_\_\_\_\_  
 70  
 His - Ile - Asp - Ser - Gln - Lys - Ala - Ile - Glu - Arg - Met - Lys - Asn -  
 \_\_\_\_\_  
 80  
 His - Ile - Asp - Ser - Gln - Lys - Ala - Ile - Glu - Arg - Met - Lys - Asn -  
 \_\_\_\_\_  
 90  
 His - Ile - Asp - Ser - Gln - Lys - Ala - Ile - Glu - Arg - Met - Lys - Asn -  
 \_\_\_\_\_  
 100  
 His - Ile - Asp - Ser - Gln - Lys - Ala - Ile - Glu - Arg - Met - Lys - Asn -  
 \_\_\_\_\_  
 103

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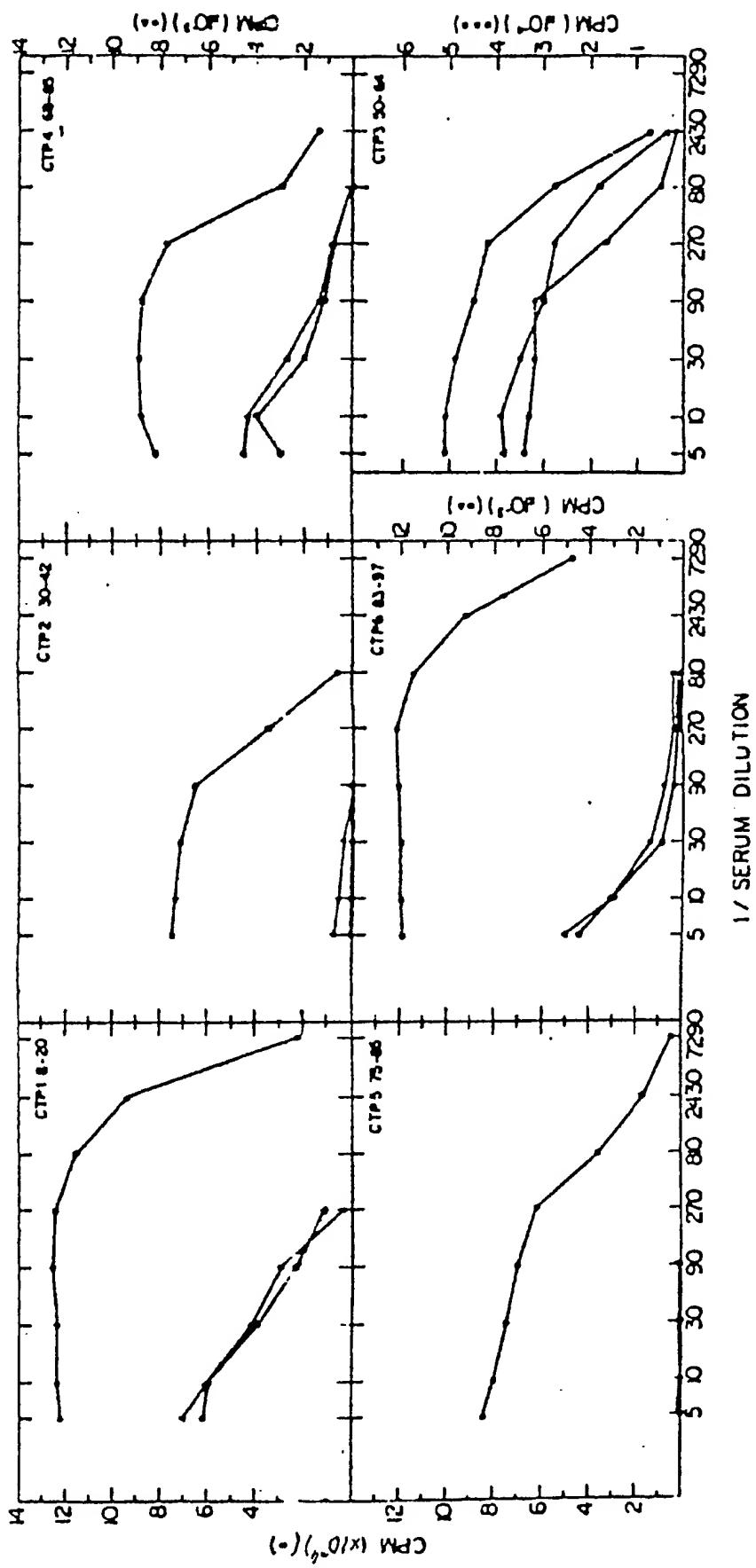


Fig. 2

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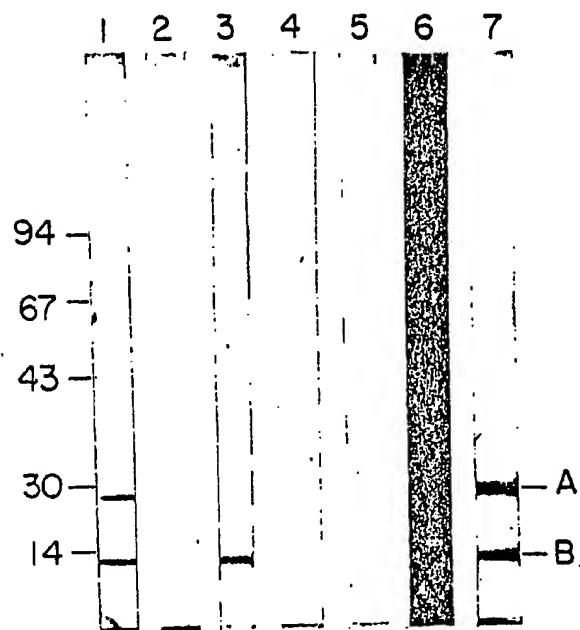


Fig. 3

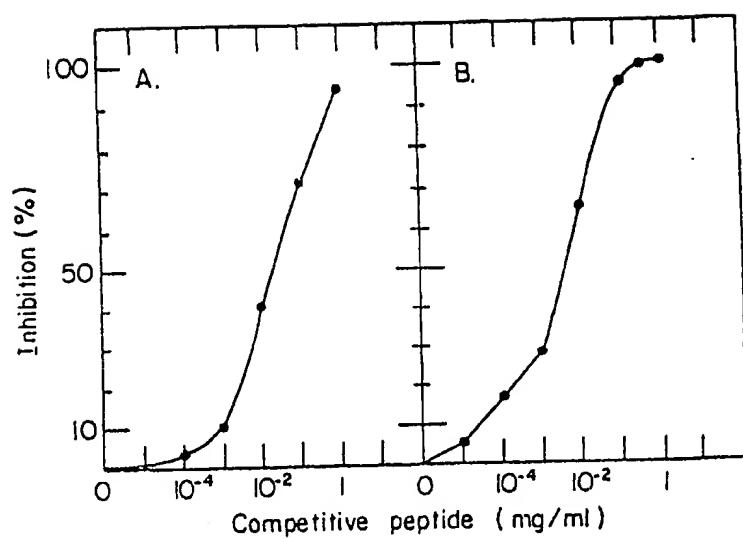


Fig. 4

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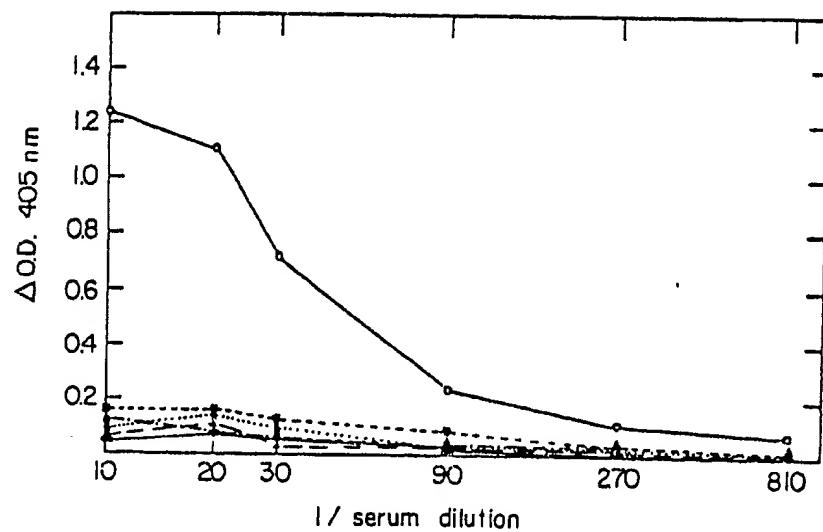


Fig. 5

## SPECIFICATION

## Vaccines

5 The invention relates to vaccines, adapted to induce selective immunity against cholera toxins (CT) and  
against heat labile toxin of E. Coli (LT of E. Coli). The vaccines are based on a combination of a  
high-molecular-weight moiety conjugated with a synthetic polypeptide which corresponds to a certain  
relatively short part of the B subunit of natural toxin. Polypeptides of choice are those corresponding to  
sequence 50 to 64, 8 to 20 and 45-64 of the natural B subunit of cholera toxin. The carrier can be a suitable  
10 toxoid, such as tetanus toxoid, or a synthetic polymeric carrier of adequate molecular weight, such as PolY  
AL (an alanine-lysine polymer) of a molecular weight of at least about 50,000 and preferably in the 100,000  
range. The polypeptide can be synthesized by conventional methods of polypeptide synthesis such as solid  
phase synthesis according to Merrifield. 5

15 Antisera to the sequence 50 to 64 neutralize in a significant manner the biological activity of cholera toxin  
and also of heat labile toxin of E. Coli. 10

Suitable synthetic peptides, attached to appropriate carriers, lead to antibodies capable of cross-reacting  
with proteins containing the peptides in their sequences. This has been shown to be true for hen egg-white  
lysozyme, for the coat protein of the bacteriophage MS2, and for influenza hemagglutin. Similar results have  
been reported on protein M of Streptococcus pyogenes, diphtheria toxin hepatitis virus, and foot and mouth  
20 disease virus. In several cases of viruses and toxins, the antibodies formed were capable of neutralizing the  
biological activities. 15

The purpose of the present invention is to provide synthetic peptides capable of provoking antibodies  
neutralizing efficiently cholera toxin and other similar toxins, such as heat-labile toxin of E. Coli. The toxin of  
Vibrio cholerae is composed of two subunits, A and B. Subunit A activates adenylate cyclase which triggers  
25 the biological activity, whereas subunit B is responsible for binding to cell receptors, and expresses most  
immunopotent determinants. Antibodies to the B subunit are capable of neutralizing the biological activity of  
the intact toxin. The B subunit (choleragenoid) is a pentamer, each of the chains containing 103 amino acid  
residues. 20

The invention relates to the synthesis of several peptides derived from the B subunit of the cholera toxin,  
30 and to antibodies neutralizing the intact cholera toxin as well as other toxins, such as heat-labile toxin of E.  
Coli. 25

There are provided synthetic vaccines against cholera toxins and against heat-labile toxin of E. Coli. These  
comprise a suitable polypeptide, corresponding to a predetermined part of the B subunit of cholera toxin,  
conjugated to a suitable polymeric high molecular weight carrier, such as a suitable toxoid (tetanus toxoid or  
35 the like) or to a suitable polymer, such as PolY AL of a molecular weight of at least about 50,000 and  
preferably in the 100,000 to 120,000 range. The synthetic polypeptides of choice are either the one  
corresponding to sequence 50 to 64, to the sequence 45 to 64, or to sequence 8 to 20 of the B subunit of  
cholera toxin. The 50 to 64 sequence unit has the composition, Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-  
40 Gln-Lys-Lys-Ala, while the 8 to 20 sequence has the composition, Leu-Cys-Ala-Glu-Tyr-His-Asn-Thr-Gln-Ile-  
His-Thr-Leu. The sequence 45-64 has the composition, Gly-Ala-Thr-Phe-Glu-Val-Glu-Val-Pro-Gly-Ser-Gln-  
His-Ile-Asp-Ser-Gln-Lys-Lys-Ala. In the 50 to 64 sequence it is possible to substitute instead of the N-terminal  
Val any other amino acid. Instead of the C-terminal Ala there can be inserted Cys, and such modified  
polypeptides will have a similar activity, or even a more pronounced one. In the 8-20 sequence there can be  
45 inserted instead of Cys the Ala amino acid and a similar activity is obtained. This was established by  
experimental work. 40

It is clear that slight modifications of the above defined sequences can be resorted to, both as regards  
chain length and composition, without departing from the scope and spirit of the invention. The polypeptide  
must be conjugated to a suitable carrier of adequate molecular weight in order to be able to induce antibody  
formation. Tetanus toxoid has been found to be a suitable carrier. There may also be used any suitable  
50 polymer, preferably based on amino acids, such as PolY AL (an alanine-lysine copolymer) of the range of  
average M.W. of about 100,000 to 120,000. It seems that a MW of at least 50,000 is required. 45

The invention is illustrated in the following with reference to certain specific embodiments, which ought to  
be construed in a non-limitative manner.

The synthetic vaccines have been found to be effective also against heat-labile toxin of E. Coli (LT of E.  
55 Coli).

Antibodies against the two peptides neutralize the activity of LT of E. Coli in the same manner as cholera  
toxin (CT). The third polypeptide, of sequence 45 to 64 has a slightly better activity than that of sequence  
50-64. There was tested the cross-reaction of anti(8-20-polypeptide) and anti-(50-64-polypeptide) with LT of  
E. Coli in a radioimmunoassay and in an immuno-blotting test; they were found to be cross-reactive.

60 Neutralization of LT-E. Coli prevents adenylate cyclase activity induced by the toxin. It prevents secretion  
of fluid into ligated ileal loops of rat intestine; it prevents cholera toxin induced adenylate cyclase activity.

Cholera toxin was purchased commercially. Separation of subunits and isolation of the B subunit were  
performed by gel filtration of Sephadex G-75 in 5% formic acid, according to Lai, C.Y. (1980) CRC Critical  
Reviews in Bioch 9, 171-207, t-Butyloxycarbonyl (t-boc) derivatives of the various amino acids were  
65 purchased commercially. All other reagents were of analytical grade or the best grade available. 60

Peptides were synthesized by solid phase method according to Merrifield, R.B. (1965) *Sci 150*, 178-185. The side chain protecting groups of the t-boc derivatives were as follows: benzyl ethers for the hydroxyls of serine and threonine, dichlorobenzyl ether for the phenolic hydroxyl of tyrosine, and carbobenzoxy for the ε-amino group of lysine. Asparagine and glutamine were protected at the α-carboxy group by p-nitrophenyl ester. The nitroguanidino group of arginine and the imidazole group of histidine were protected by tosyl. The initial amino acid-resin was prepared by esterification of the relevant t-boc-amino acid to chloromethylated resin (polystyrene-1% divinylbenzene). 5

The progress of synthesis was monitored by ninhydrin analysis. Two cycles of coupling were performed whenever coupling reaction was less than ≥ 99% complete by ninhydrin test and amino acid analysis. For 10 the synthesis of CTP 65% (v/v) 1,2-ethanedithiol was added to the trifluoroacetic acid to prevent oxidation of tryptophan. The protecting groups were removed and the peptides were cleaved from the resin at 0°C with anhydrous hydrogen fluoride containing 10% anisole and 1% 1,2 ethanedithiol as scavengers. 10

Crude peptides recovered after cleavage from the resin were purified on Sephadex G-25 column. Purity of peptides was analysed by amino acid analysis, by reverse-phase HPLC and/or high voltage paper electrophoresis. 15

#### *Conjugation of Peptides with Tetanus Toxoid*

Two methods of conjugation were used:  
 I) 1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride as the coupling agent as described 20 previously, Müller et al. (1982) *Proc. Nat. Acad. Sci. USA 79*, 569-573.  
 II) While still on the resin, the peptide was elongated by t-boc-p-aminophenylacetic acid (PAPA) prior to the HF cleavage. PAPA peptides were dissolved in cold 2N HCl, diazotized by addition of ice cold aqueous sodium nitrite (0.1 M), and added to a solution of tetanus toxoid in NaHCO<sub>3</sub>(0.5 M), while maintaining pH 8.5 by addition of concentrated Na<sub>2</sub>CO<sub>3</sub>. After 10 hours at 4°C, the mixture was dialysed against 10 mM 25 ammonium carbonate and lyophilized. The peptide content of the conjugates was determined by amino acid analysis and by trace labelling with <sup>125</sup>I, when possible. 25

#### *Immunization procedure*

Rabbits were immunized by multisite intradermal injections of 1 mg of conjugate dissolved in 0.5 ml of 30 Pi/NaCl and emulsified in 0.5 ml of complete Freund's adjuvant, with several boosters, as described previously, Müller et al. (1982) *Proc. Nat. Acad. Sci. USA 79*, 569-573. *Solid Phase Radioimmunoassay (RIA)* was performed on antigen-coated (0.5 - 1.0 µg/well) V-bottom flexible microtiter plates ( precoated with glutaraldehyde (0.2%) whenever peptides were used as antigen) by addition of three-fold serial dilution of the tested serum, followed by <sup>125</sup>I protein A labelled by Bolton and Hunter reagent (10<sup>5</sup> cpm/50 µl/well). The 35 washed and dried wells were cut and counted in a gamma counter. 35

In competitive inhibition assays the antigen coated wells were incubated with 10 fold serial dilutions of the tested inhibitor peptide solution in Pi/NaCl containing 0.1% BSA, prior to addition of a constant dilution of the antipeptide serum.

40 *Enzyme labelled immunosorbent assay (ELISA)* was carried out similarly to the RIA, except that flat bottom plates were used, and a β-galactosidase conjugate of protein A (Amersham) was used instead of the radioactive label. After addition of the substrate (O-nitrophenyl-β-D galactopyranoside), the plates were read in an automatic reader. 40

45 *Immunoprecipitation*  
 Cholera toxin was labelled with <sup>125</sup>I by the chloramine-T method. Immunoprecipitation was performed basically according to Kessler Heitmancik et al., (1977) *Infect. Immun.*, 17, 621-628. Immunoprecipitation was performed basically according to Kessler, S.W. (1975) *J. Immunol.* 115, 1617-1624, with slight modifications. <sup>125</sup>I-Labelled cholera toxin was preadsorbed on intact *Staphylococcus A* and then reacted with various 50 antisera. The precipitates obtained after addition of fixed *Staphylococcus A* were analyzed by 5-15% NaDODSO<sub>4</sub> polyacrylamide gel electrophoresis and visualized by autoradiography. 50

#### *Electrophoretic blotting procedure*

Cholera toxin, separated into its subunits on a 5-15% NaDODSO<sub>4</sub> poly-acrylamide gel, was transferred to 55 nitrocellulose sheet according to the method of Towbin et al. (1979) *Proc. Natl. Acad. Sci. USA 76*, 4350-5354. To reduce nonspecific binding of antiserum, the blot was incubated for 1 hr with 9 mM Tris HCl buffer, pH 7.4, containing 0.9 M NaCl and 3% w/v BSA, and then cut into strips. The strips were incubated for 3 hr at room temperature with 1:50 dilution of different antisera.

After thorough washing, the strips were incubated for 2 hours with <sup>125</sup>I-labelled goat anti-rabbit IgG (5 × 60 10<sup>5</sup> cpm/ml). The washed and dried blots were autoradiographed. 60

#### *Toxin neutralization activity*

#### *Vascular permeability assay*

The assay was performed essentially according to Craig, J.P. (1966) *J. Bacteriol.* 92, 795, and Lai, C.Y. 65 (1980) *CRC Critical Reviews in Biochem.*, 9, 171-207 with slight modifications: 65

Serial dilutions of the test antisera were mixed with a constant amount of cholera toxin, incubated for 1 hour at room temperature and 0.1 ml of the mixtures were injected intradermally in triplicates into the shaved skin of adult rabbit. After 18 hours the rabbit was injected I.V. with 1 ml/kg of 5% Evans Blue and the diameter of the resulting blue induration was measured 1 hour later. Neutralization end point was taken to 5 be the highest dilution of serum which prevented the blueing phenomenon. Both positive (no antiserum) and negative (no cholera toxin) controls were included in each rabbit.

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### *II) The ligated ileal loop assay*

The assay was performed essentially as described by Fujita and Finkelstein (1972) J. Infect. Dis. 125, 10 647-655. Briefly, rats or adult rabbits fasted for 12 hours were anesthetized with ether, the abdomen opened, and the small intestine ligated in 3-4 cm long loops starting approximately 10 cm from the duodenum. The loops were injected with different dilutions of the tested sera previously incubated with a constant amount of cholera toxin, and the abdomen closed. Food and water were withheld and the animals sacrificed after 5 hours. Fluid accumulation per centimeter of loop was determined by measuring the length and weight of 15 each loop. Both positive (no antiserum) and negative (no cholera toxin) controls were included in each animal.

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### *Cleavage of B subunit*

To locate the regions participating in antigenic reactivity, the B subunit was cleaved by CNBr to yield three 20 fragments with the following sequences: 1-37 and 69-101 linked by a disulfide bridge between cysteine residues at positions 9 and 86; 36-68 and 102-103. The fragments were separated on a Sephadex G-50 column, and the two larger peptides were tested for reactivity with antisera prepared against cholera toxin. The largest peptide was partially cross reactive with anti cholera toxin sera, whereas the peptide 38-68, although incapable of binding directly to the antisera, was capable of inhibiting the toxin-anti-toxin 25 homologous anti-sera (results not shown).

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### *Selection of peptides for synthesis*

Considerations based on the above results obtained with the cyanogen bromide fragments of B subunit, dictated the selection of peptides for chemical synthesis. Peptide 30-42 (CTP 2) was synthesized since it was 30 suggested that Arg 35 or the region surrounding it may be involved in antibody and receptor binding activity, Duffy et al. (1979) Biochem. Biophys. Res. Comm. 91, 1005-1010. Peptide 83-97 (CTP 6) containing tryptophan at position 88 was synthesized, since chemical modification of this residue had resulted in loss of GM1 binding to cholera toxin, De Wolf et al. (1981) J. Biol. Chem. 256, 5481-5488. We have also synthesized the peptide containing the highest local average hydrophilicity (residues 79-84), since such sequences are 35 thought to be located in or immediately adjacent to antigenic determinants, Hopp et al. (1981) Proc. Natl. Acad. Sci. USA 78, 3824-3828. Based on the reported role of tyrosine residues for antigenicity, Arnon et al. (1960) Biochem. J. 75, 103-109, peptides containing, respectively, Tyr 12 and Tyr 76, namely, CTP 1 (residues 8-20) and CTP 5 (residues 75-85) were included. Since the latter peptide contained only 11 residues, we prepared an additional peptide with a longer sequence (residues 69-85, denoted CTP 4). Peptide 50-64 (CTP 40 3) was synthesized since it is a part of the inhibitory CNBr fragment 38-68.

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### *Synthesis and conjugation of peptides*

The peptides synthesized correspond to several regions of the B subunit of cholera toxin (Figure 1). The only change from the native sequence was the replacement of cysteines at position 9 and 86 (in CTP 1 and 6), 45 respectively, by alanine to avoid formation of aggregates. The results of amino acid analyses of the peptides were in good agreement with the expected values for the various amino acid residues. The purity of the peptides was further established by reverse phase HPLC and/or high voltage paper electrophoresis, indicating less than 5% impurities in the end products. The purified peptides were conjugated to tetanus toxoid either using a water-soluble carbodiimide as a coupling agent, or through an azo bond, when PAPA derivatives of the peptides were employed. The advantage of binding via PAPA residue is the specificity of 50 the conjugation which occurs only between the N-terminal amino group of the PAPA and histidine or tyrosine residues on the carrier, Spirer et al. (1977) Eur. J. Immunol. 7, 69-74. Both methods of coupling yielded adequate conjugates (Table 1).

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### *55 Immunological reactivity*

The conjugates of tetanus toxoid with all six peptides induced antibodies specific towards the respective homologous peptide (Figure 2). As shown, the highest response was observed with CTP 1 and CTP 6. Four of the antisera were also cross-reactive to a different extent with the intact B subunit and whole native cholera toxin. This cross-reactivity was demonstrated by three assays - by solid phase radioimmunoassay (Figure 2), 60 immuno-blotting technique (Figure 3) and by immunoprecipitation (Table 2). All three methods showed similar results.

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Peptides CTP 3 induced antibodies which gave a very strong cross-reactivity with the intact toxin, similar in its level to that of the homologous peptide anti-peptide reaction (Figure 2). Both the homologous peptide anti-peptide reaction and the cross-reactivity are indeed specific, since they can be completely inhibited by 65 excess of the free CTP 3 peptide (Figure 4). Furthermore, CTP 3 is the only peptide of those investigated

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which reacted with antiserum against the intact native cholera toxin (Figure 5). Thus, the cross-reactivity with a native protein is not a characteristic of just any peptide segment derived from it.

This is further confirmed by a comparison of the related peptides CTP 4 (69-85) and CTP 5 (75-85).

Antiserum against CTP 5 was incapable of cross-reacting with either B subunit or whole toxin, although it had a high titer towards the homologous peptide. Elongation of this peptide by 6 amino acid residues resulted in CTP 4, which elicited antibodies cross-reactive with the intact proteins, even though the homologous anti-peptide titer was not significantly higher than in the case of CTP 5. 5

The results of the immunoprecipitation experiment (Table 2) present the quantitative aspect of the cross-reactivity between the anti-peptides and cholera toxin. The anti-peptide CTP 3 indeed gives the highest 10 cross-reactivity, amounting to about 30 percent of the homologous toxin-anti toxin reaction. In accordance with the results of the RIA, anti CTP 1 and CTP 6 are significantly cross-reactive with the intact toxin, whereas the remaining peptides show only slight reactivity. The immunoblotting experiment serves as another confirmation of these results, and raises an additional point of interest: since in this case the intact cholera toxin was separated by electrophoresis into its two subunits prior to the interaction with the various antisera, 15 it is clear from the results (Figure 3) that antiserum to CTP 1 reacts not only with the B subunit, but also, to an appreciable extent, with the A subunit of cholera toxin. 15

#### *Inhibition of adenylate cyclase activity*

Since the diarrhea occurring in response to CT or LT is a result of activation of the adenylate cyclase in the 20 cell membrane of small intestinal epithelial cells, we have evaluated the capacity of the different antisera to inhibit CT-induced or LT-induced adenylate cyclase activity. The enzyme activity was determined by testing the level of cAMP production in dispersed kidney cells of chickens. As shown in Table 5, antisera to CTP1 and CTP3 specifically inhibit CT-induced adenylate cyclase activity up to about 60-65%. The same is true for CT induced adenylate cyclase activity, namely CTP1 and anti CTP3 specifically inhibit its activity to a similar 25 extent. 25

#### *Neutralization of biological activity*

The sera against the various peptides were evaluated for their capacity to neutralize biological effects of cholera toxin. For this purpose, two *in vivo* assays of the cholera toxin activity, namely, a skin test measuring 30 the increased vascular permeability induced by the toxin, as well as the fluid accumulation induced by cholera toxin in ligated small intestinal loops of adult rabbits, were employed. The results of these two experiments are presented in Tables 3 and 4. In both assays anti-CTP 3 caused partial inhibition of the toxin activity. Although less effective than antiserum against native cholera toxin, the anti-CTP 3 reduced quite significantly - up to 40% - the fluid accumulation in rabbit intestine. These two assays, although performed *in vivo*, demonstrate only the presence of antibodies in the sera of the immunized rabbits. Peptide CTP 3, which 35 showed the strongest immunological cross-reaction with cholera toxin, has an appreciable ability to induce antibodies capable of neutralizing the biological activity of cholera toxin. It can be used for inducing protective immunity towards the toxin. 35

The above experiments indicate that the synthetic vaccines thus produced can be used for vaccination 40 against cholera toxin. The required quantity of the polypeptide-carrier conjugate for vaccination of humans is in the range of about 2 to 20 mg. 40

It is clear that the conjugate has to be administered in a suitable carrier with suitable adjuvants.

Reference is made to the enclosed drawings, in which:

Figure 1 provides the amino acid sequence of subunit B of cholera toxin; 45  
Figure 2 illustrates the antibody response of rabbits to different peptides of subunit B of cholera toxin;  
Figure 3 illustrates the cross-reactivity of different peptides established by the immunoblotting technique;

Figure 4 illustrates the cross-reactivity of the peptides and the inhibition by one of the synthetic peptides;  
Figure 5 illustrates the reactivity of the 50-64 peptide with antiserum against cholera toxin.  
50 In the above Figures Figure 2 illustrates the antibody response of rabbits to different peptides of the B subunit of cholera toxin. Reactions with: homologous peptides (solid circles); the B subunit of cholera toxin (triangles); and cholera toxin (rings). Note the difference in the scales of the reactions with the peptide and the intact proteins in the case of all peptides except CIP 3. 50

TABLE 1

*Properties of various peptide conjugates with tetanus toxoid (MW 150,000)*

	Peptide	Position	Method of coupling	used for coupling (mol/mol)	Peptide / carrier ratio in the conjugate (mol/mol)	
10	BP <sub>1</sub>	8-20	PAPA*	95	54	10
			EDCI**	40	27	
15	BP <sub>2</sub>	30-42	EDCI	45	11	15
	BP <sub>3</sub>	50-64	PAPA	94	63	
20	BP <sub>4</sub>	69-85	EDCI	41	14	20
				40	19	
	BP <sub>5</sub>	75-85	EDCI	35	10	
	BP <sub>6</sub>	83-97	PAPA	75	25	
			EDCI	49	24	

\*PAPA = p-Aminophenylacetic acid, attached to the peptide via an amide bond, and coupled to the protein after diazotization,

\*\*EDCI = 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

TABLE 2

	Immunoprecipitation of <sup>125</sup> I-Cholera toxin by different antipeptide sera			
	Serum sample	<sup>125</sup> I-Cholera toxin precipitated cpm	% of total radioactivity	
30	anti CTP1	41880	4.4	30
	anti CTP2	9119	0.9	
	anti CTP3	66498	7.0	
	anti CTP4	4956	0.5	
35	anti CTP5	2980	0.3	35
	anti CTP6	47813	5.0	
	anti cholera toxin	221560	23.0	
	preimmune serum	2694	0.28	

TABLE 3

*Inhibition by anti CTP 3 peptide of cholera toxin-induced vascular permeability in rabbit skin*

45	Challenge (ng toxin)	Vascular permeability reaction <sup>a</sup>			45
		anti cholera toxin	anti CTP 3 <sup>b</sup>	preimmune serum <sup>b</sup>	
50	0.5	—	—	+++	
	1.0	—	+	+++	50
	2.0	—	+++	+++	
	3.0	—	+++	+++	

<sup>a</sup> +++ strong blue induration

+ faint blue induration

— no blue colour

<sup>b</sup> All sera were used at 1:10 dilution

TABLE 4  
*Neutralization of cholera toxin by antisera to peptide CTP 3 (50-64)*

	<i>Cholera toxin</i> ( $\mu$ g)	<i>Serum</i>	<i>Dilution</i>	<i>Weight/cm loop</i> (g)	<i>Ligated ileal loop</i>	<i>Reduced secretion<sup>a</sup></i> (%)	
10	0.0	none (saline)		0.20			10
	1.5	none (saline)		1.30			
	1.5	anti cholera toxin	1:20	0.45			
	1.5	anti-CTP 3	1:2	1.02		40	
15	1.5	normal rabbit serum	1:2	1.25			15
	3.0	none (saline)		1.36			
	3.0	anti cholera toxin	1:20	0.51			
	3.0	anti-CTP 3	1:2	1.08		33	
20	5.0	none (saline)		1.42			20
	5.0	anti cholera toxin	1:20	0.58			
	5.0	anti-CTP 3	1:2	1.32		12	
25	7.5	none (saline)		1.42			25
	7.5	anti cholera toxin	1:20	0.60			
	7.5	anti-CTP 3	1:2	1.32		12	
	10.0	none (saline)		1.50			
30	10.0	anti cholera toxin	1:20	0.69			30
	10.0	anti-CTP 3	1:2	1.50		0	

<sup>a</sup>Assuming that the reduction effected by antiserum to cholera toxin is 100%.

TABLE 5  
*Specificity of cAMP inhibition by antisera*

40	<i>Antiserum added</i>	<i>Cyclic AMP induced by:</i>						40	
		<i>Cholera toxin</i>		<i>Parathyroid hormone</i>		<i>Isoproterenol</i>			
		<i>pmoles/ 250000 cells</i>	<i>%</i>	<i>Significance (p)<sup>a</sup></i>	<i>pmoles/ 250000 cells</i>	<i>%</i>	<i>pmoles/ 250000 cells</i>	<i>%</i>	
45	None	5.9 <sup>b</sup>	100		3.7	100	4.8	100	45
	NRS	5.6	95	n.s. <sup>c</sup>	3.5	94	4.6	95	
50	CT	0.5	8	0.001	3.8	100	4.6	95	50
	CTP1	3.2	54	0.01	3.7	100	4.7	98	
	CTP3	2.5	42	0.01	3.5	94	4.6	95	
	TT	5.6	95	n.s.	n.t. <sup>d</sup>		n.t.		

55 a Significance of the difference between cholera toxin induced cAMP without antiserum and with any of the antisera used. 55

b Values represent mean of 3 experiments.

c n.s. - Not significant

d n.t. - Not tested.

## CLAIMS

1. A synthetic vaccine against cholera and against heat-labile toxin of E. Coli, comprising a conjugate of a high-molecular weight carrier with a synthetic polypeptide corresponding to a part of the sequence of Subunit B of natural cholera toxin with a suitable adjuvant or diluent. 5

2. A vaccine according to claim 1, wherein the sequence corresponds essentially to that of sequence 50 to 64 of the cholera toxin. 5

3. A vaccine according to claim 1, wherein the polypeptide has essentially the sequence, Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile Asp-Ser-Gln-Lys-Lys-Ala. 10

4. A vaccine according to claim 1, wherein the sequence corresponds essentially to 8 to 20 of Subunit B of cholera toxin. 10

5. A vaccine according to claim 4, wherein the sequence corresponds essentially to Leu-Cys-Ala-Glu-Tyr-His-Asn-Thr-Gln-Ile-His-Thr-Leu. 15

6. A synthetic vaccine corresponding essentially to sequence 45 to 64 of the cholera toxin. 15

7. A synthetic vaccine according to claim 6, wherein the sequence corresponds essentially to Gly-Ala-Thr-Phe-Glu-Val-Glu-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-Gln-Lys-Ala. 15

8. A vaccine according to any one of claims 1 to 7, wherein the high molecular weight carrier is a toxoid. 20

9. A vaccine according to claim 8, wherein the toxoid is tetanus toxoid.

10. A vaccine according to claim 8, wherein the high molecular weight carrier is a polymer of the type POLY AL of an average molecular weight of at least 50,000. 20

11. A synthetic vaccine according to any one of claims 1 to 10 when produced by a method substantially as hereinbefore described. 20

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